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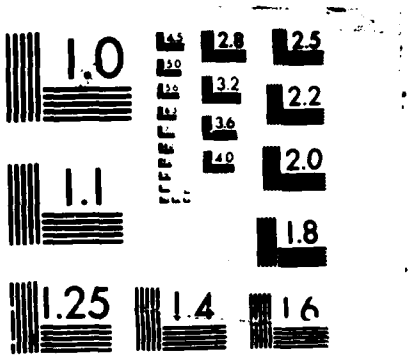

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The thrust of this project is to develop and apply new methodologies based on nonlinear optics to the study of biological macromolecules and membranes. In the period covered by this report we developed several biochemical experimental systems to which the spectroscopic techniques are being applied. The biological problems addressed include: a. Age-related modifications in enzyme catalysis. 2. Mechanisms of control of membrane-bound transport proteins. The spectroscopic studies involved two primary sets of measurements based on backward nearly degenerate four-wave mixing and employing both time domain and frequency domain measurements. A CW dye laser system using cross-correlated optical fields was applied to measure relaxation rates of nonradiating states like nonphosphorescent triplet states and is being adapted for studies of biological systems. A laboratory for ultrafast time-domain measurements is being developed to study enzyme and membrane mechanisms. A system based on phase conjugate optics designed to eliminate the effects of light scattering in optical spectroscopy studies of biological membrane systems is being tested.

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# ANNUAL REPORT

## NONLINEAR LASER SPECTROSCOPY STUDIES OF ENZYME INTERACTIONS

Contract No. N00014-86-K-0124

Period Covered: 2/1/86-2/1/87

Co-Principal Investigators: A. Gafni and D. G. Steel

University of Michgian

## I. PROGRAM OBJECTIVES AND APPROACH

The primary emphasis of this program has been to develop and apply methodologies based on nonlinear optics to the study of biological macromolecules. During the first year of research, we have developed several experimental systems which are both of great biological interest and excellent systems for nonlinear spectroscopy studies. The projects address two different problems: 1. Age-related modifications in enzyme catalysis; and 2. Mechanisms of control of membrane-bound transport proteins. The primary optical methods are based on time and frequency domain nonlinear laser spectroscopy and also on time resolved emission and optical phase conjugation to eliminate the effects of scattering in spectroscopy and imaging.

Nonlinear laser spectroscopy studies of materials are based on the frequency and time domain response of the third order optical susceptibility. The objective of such measurements is to study the interaction between a specific resonant system which is coupled to the light and the surrounding environment. In particular, one of the measurements we are working on is studying the relaxation properties of a chromophore (a dye molecule used as a label) attached to a specific location in the active site of an enzyme. By studying the various relaxation rates associated with a state or coherent superposition state of the chromophore, we anticipate being able to study changes in the active site.

Another problem is that many enzymes in their native state are bound in membranes which cause scattering of the incident beam. In addition, scattering by these membranes make imaging of structures very difficult. One of the objectives in this program is to use techniques based on optical phase conjugation and ultrafast time resolved emission to reduce the effects of scattering on spectroscopy and imaging.

## II. PROGRESS

Following construction of the first part of the new laboratory, we have begun two primary sets of measurements based on backward nearly degenerate four-wave mixing. In these experiments, two counter propagating pump beams interact with a probe beam in the sample to generate a spatially and temporally coherent signal which is counterpropagating with respect to the probe beam. The signal is detected and averaged with a high speed computer system. The frequency of each of the input beams can be independently varied. By controlling the polarization of the different beams, effective longitudinal or transverse relaxation rates can be measured.

In the first set of measurements, we have begun studying the NDFWM response of dyes which we anticipate will be useful for labeling and for comparison to time domain measurements. These measurements may also be sensitive to solvent contributions to the nonlinearity, so we have studied the nonlinear response in water and methanol. Frequency domain measurements of relaxation times of the order of a few picoseconds have been observed. Figure 1 shows an example of the four-wave mixing spectrum obtained for the dye Nile Blue in methanol. The central feature is due to either thermal effects or population dynamics and has a width determined by the convolution of the two dye lasers. However, broad background is due to rotational and vibrational relaxation as well as possible relaxation contributions from coupling to surrounding solvent shells.

In a separate set of measurements, we have used a cw dye laser system and the method of cross-correlated optical fields to measure relaxation rates of nonradiating states such as triplet states with undetectable phosphorescence. Such measurements have been made by our group in solids, but this was the first effort to make such measurements in liquids. In biological systems, these measurements are extremely valuable in providing a means to study protein structure changes which result in a change in the amount of triplet-singlet mixing. We have measured several relaxation times resulting in backward nearly degenerate four-wave mixing linewidths of several hundred hertz. Figure 2 shows an example of our data. The insert is data taken earlier in $CdS_{1-x}Se_x$ which demonstrates the technique and shows the relaxation of a nonradiating deep level trap. The main structure in the figure is the NDFWM spectrum obtained in LDS722. However, we have determined that the response in the liquid is dominated by a new kind of optical nonlinearity which we believe is due to convective changes in the index. Our current work is based on eliminating this contribution from the signal, and simultaneously developing an alternative approach based on nonlinear absorption measurements in the time domain.

For the first time, we have also determined that we have detected alignment signals using low power cw excitation. These results are extremely encouraging for providing sensitive measurements of dephasing of coherent superposition states.

In parallel with this work, we are developing a laboratory for ultrafast time domain measurements. Time and frequency domain measurements are in principle related by their Fourier transform. However, complicated systems such as enzymes in solution are not anticipated to follow such a simple relationship. Using a short pulse laser, we are working on developing other spectroscopy techniques such as photon echos for measurement of additional relaxation effects.

Finally, nonlinear optical methods are also useful for enhancing linear spectroscopy methods in turbid systems. The problem is similar to attempting to study an object surrounded by glass marbles. Scattering complicates the interpretation of spectroscopic signals. To study enzymes which are bound in membranes, we are working on a method based on optical phase conjugation to eliminate scattering effects. On a separate program, we have made the first demonstration of a near real time optical phase conjugate mirror with a wide field of view. We are currently designing a system to incorporate this mirror in a spectroscopy experiment. In earlier work we, along with other groups, have demonstrated that this method is extremely effective in eliminating the effects of scattering due to index fluctuations with characteristic scale

lengths that are long compared to a wavelength. However, membrane vesicles have dimensions of the order of 1 micron. This generates an angular tolerance for the counter propagating pump beams of order $10^{-4}$ rad.

Along similar lines, we are also examining the possibility of using ultrashort pulsed lasers to permit temporal discrimination between scattered light (long time) and spectroscopy signals (obtained on short time.)

A major development in our biochemical studies was our recent demonstration that the age-related effects in rat muscle phosphoglycerate kinase (PGK) may be successfully reversed by a complete unfolding of the polypeptide chain in 2 M guanidine: HCl followed by refolding under mild conditions. Samples of old PGK, thus treated, become identical with young PGK in their heat inactivation patterns as well as in their refolding kinetics. This is the first demonstration that age-related effects at the molecular level may be reversed in vitro and is a finding of major significance. Moreover, this rejuvenation of old PGK clearly shows that the aging effects in this enzyme are purely conformational and hence develop post-synthetically. The nature of these modifications, their location and mechanism of development in the old tissue are as yet not understood and are of major interest. We do, however, have solid evidence that the structural modifications in old PGK occur outside the active site in a domain which is involved in maintaining the three dimentional structure of the enzyme. This domain contains tryptophan residues and is therefore amenable to spectroscopic studies. We thus plan to employ the optical spectroscopic techniques discussed before in an attempt to probe with great sensitivity the local environments of the tryptophan residues as well as of extrinsic chromophores specifically bound to selected sites on the enzyme. The use of the laser system in the time domain will allow us to follow in detail the modifications introduced by old age into PGK's mechanism of catalysis. The kinetics of decay of the first triplet state, monitored by following triplet-triplet transition of tryptophan residues, will be used to assess the structural transition that occur upon unfolding, refolding and during aging of this enzyme.

In addition to PGK we have begun a study of the enzyme phosphoglycerate mutase (PGM) and our preliminary results indicate the presence of age-related modifications in this enzyme too. PGM has an unusual long-lived tryptophan emission which may serve as a useful probe for the enzyme's structural integrity. We also have indication that PGM forms a complex with PGK and this system will be studied along with the PGK: GPDH complex as part of our effort to determine the effects of old age on complex formation among glycolytic enzymes.

Membrane bound proteins: Our studies into the mechanisms of control of the sarcoplasmic reticulum (SR) calcium pump have developed rapidly and currently focus on the determination of the spatial relationship between the catalytic site and the four $Ca^{2+}$ binding sites of this enzyme. In these studies we employ diffusion-enhanced energy transfer (DEET) measurements from $Tb^{3+}$ ions to a rhodamine derivative covalently bound inside the ATP binding site. We have developed a simplified version of the DEET technique which allows the determination of molecular distances from steady state fluorescence measurements-an approach which renders this methodology much more accessible to biochemists.

We have recently initiated a study into the mechanism of the co-translocation of ATP and ADP across the mitochondrial membrane. This reaction is catalyzed by a membrane-bound protein whose mechanism of control is still largely unknown. We are studying the conformational transitions in the translocase which accompany its transport cycle. Here, again, we will use the high resolution in both time and frequency of the laser system to gain information which cannot be obtained by using classical spectroscopic techniques. Two experimental approaches which are expected to be of particular importance in the study of these membrane bound proteins are the elimination of the notorious problem of light scattering by the phase-conjugation technique and the determination of microsecond kinetics by the time-resolved triplet-triplet transitions measurements.

## III. FUTURE WORK

In the next few months, a picosecond laser system will be installed along with an ultrafast (sub-picosecond) laser system. The first system will be used with a picosecond photon counting system in fluorescence lifetime measurements and for UV generation for triplet state relaxation rate measurements. The second system will be used to make time domain measurements of decay of optical induced coherences and assist in imaging and spectroscopy in turbid systems.

Using the YAG pumped dye laser system, we will increase the accuracy of our spectroscopy measurements to improve our sensitivity to conformational changes. One of the primary objectives is to add digital phase sensitive detection to the box car integrator as well as establishing the necessary software to permit a nonlinear least squares analysis of spectral curves.

To eliminate convective contributions to the measurement of triplet state lifetimes, we will work on preparing samples in a host with much higher viscosity than water.

## Figure Legends

Fig. 1 NDFWM spectrum using pulsed lasers for Nile Blue in methanol. The dashed line represents an approximate curve fit illustrating the contribution to the line width due to orientational relaxation.

Fig. 2 Continuous wave NDFWM spectrum obtained in LDS722. The insert shows the NDFWM spectrum obtained in Cd $S_x$ $Se_{1-x}$ which provides a measurement of the lifetime of a nonradiating trap.

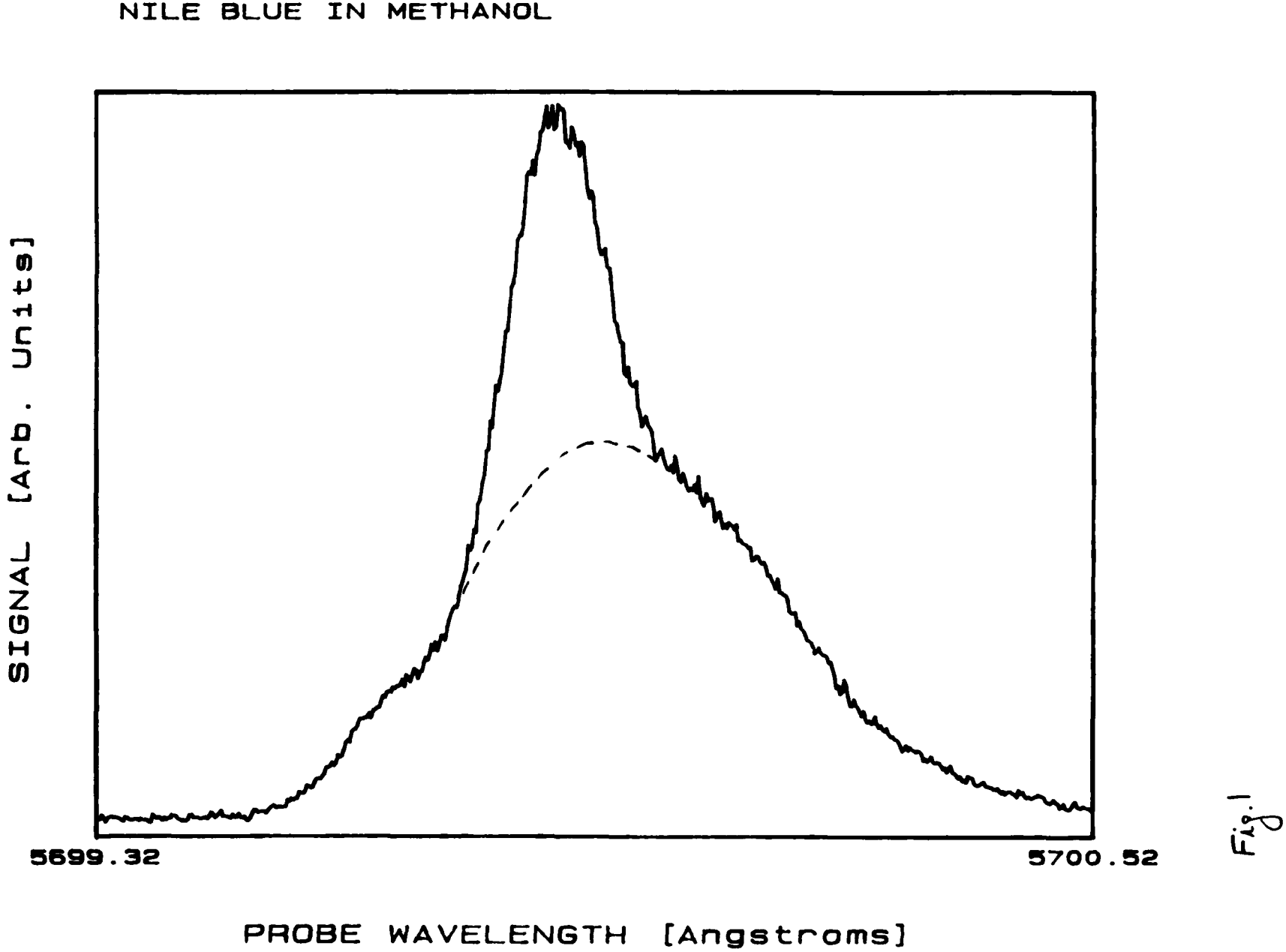
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Fig. 1

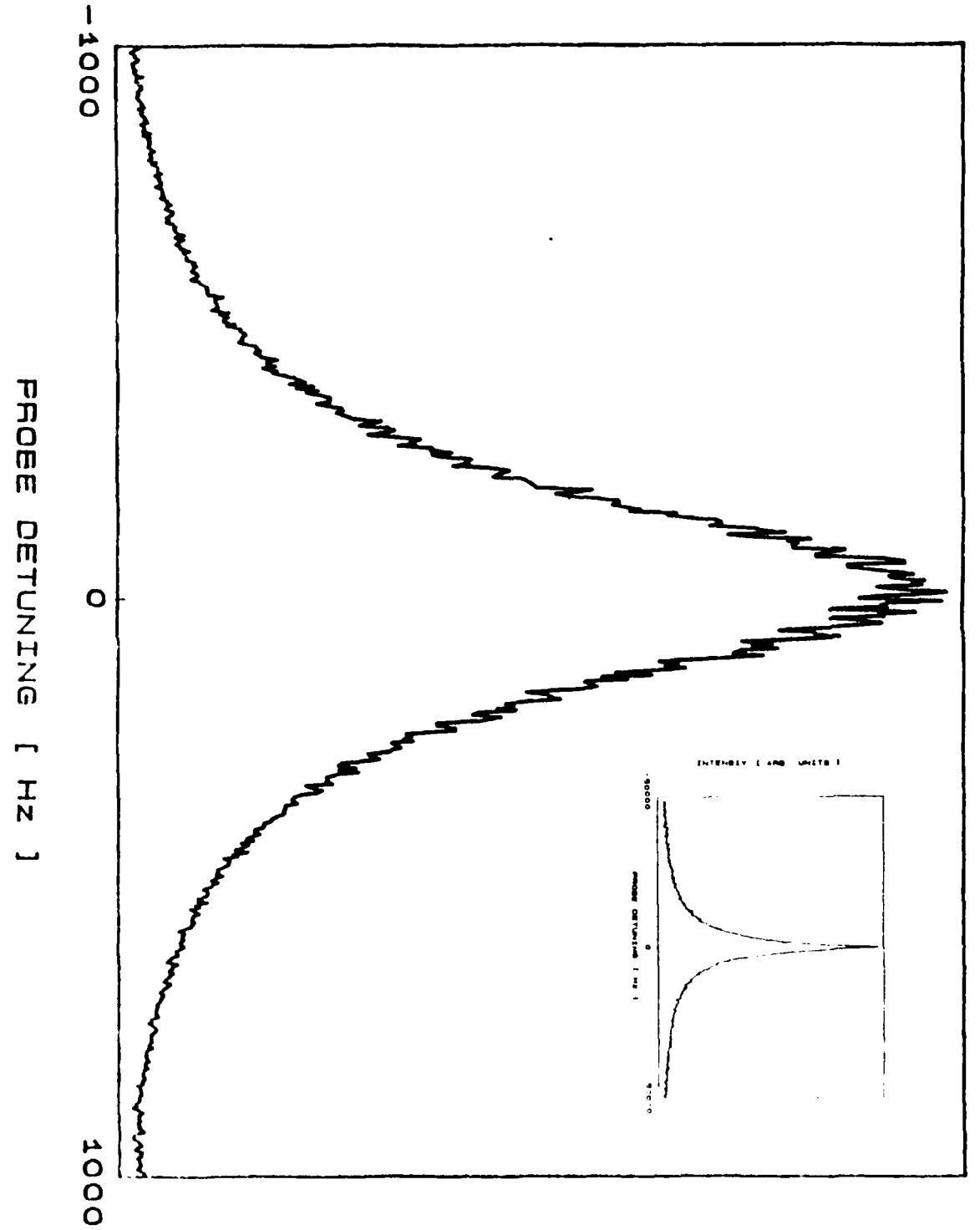


Fig. 2

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